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Isolation and Description of the Pectinase-Inhibiting Tannins of Grape Leaves

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SUMMARY

Studies were continued on the tannin in Muscadine grape leaves previously reported to have inhibitory activity toward pectinase and cellulase. The tannin was isolated and purified by precipitation with caffeine and recovery by chloroform extraction. The material is a light tan, and in the freeze-dried form is quite bulky. It is a condensed tannin of high molecular weight, producing a large percentage of phlobaphenes upon acid treatment. The soluble portion after acid treatment was shown to contain gallic acid and glucose in small amounts.

INTRODUCTION

The importance of obtaining a source of inhibitor for enzymes responsible for the softening of cucumbers during brining was discussed by Bell and Etchells (1958), Etchells *et al.* (1958a,b), Bell *et al.* (1960), and Porter *et al.* (1961). Several plants were reported by Bell *et al.* (1962) to contain varying amounts of the inhibitor active against pectinase and cellulase. Grape leaf extracts were shown by Veldhuis (1961) to have inhibitory activity against pectin esterase. The active principle was identified by Porter *et al.* (1961) as a tannin or tannin-like material. Continued work toward isolation and identification has shown the active material to be a tannin of the condensed type.

MATERIALS AND METHODS

Plant materials. Muscadine grape leaves, Scuppernong variety, collected in North Carolina in late July, 1960, were washed with water, surface dried, placed in polyethylene bags, and frozen. The samples were stored at −15°C or below.

Extractions. The frozen leaves (80 g) were divided into 40-g portions, cut with scissors, and placed in a Waring blender (no endorsement implied). Distilled water (300 ml) was added to each 40 g of leaves, and the blender was run at low speed for 30 sec and then at high speed for 2.5 min. The slurry was filtered through four layers of cheesecloth. The filtrate was refiltered several times through the filter cake produced by the coarse fibers of the leaves, until essentially clear. The two extracts were combined, yielding about 600 ml.

Isolation of tannin fraction. The tannins were isolated by the method of Barnes (1956) slightly modified. The extract was cooled to 5°C using an ice-salt bath. Caffeine (6 g) was dissolved in 400 ml of water at 55°C, and the solution temperature was lowered slightly. The caffeine solution was added slowly, with stirring, to the extract. Addition was discontinued if the temperature approached 14°C, and was continued only when the temperature was lowered to less than 7 or 8°C. After all caffeine had been added, the mixture was cooled to 5°C and allowed to stand until the precipitate had settled (about 30 min). The clear supernatant solution was decanted, and the suspension of the caffeine-tannin complex was transferred to two 250-ml centrifuge bottles. The suspension was centrifuged for 20 min at 1850–2000 rpm in an International Centrifuge, type SB, size no. 1. The supernatant solution was decanted, and the precipitate resuspended in 25 ml of water and recentrifuged. The wash solution was discarded. The two precipitates were combined and transferred to a liquid-liquid extracter using a total of 125 ml of water. The suspension was continuously extracted with chloroform until the caffeine was removed and the tannin was redissolved in the water phase (about 10 hr). The tannin solution was filtered through Whatman no. 5 paper with suction, evaporated twice to a small volume (to remove chloroform) in a rotary evaporator at a bath temperature of 35°C. The resulting solution was freeze-dried and stored in tightly stoppered bottles.

Measuring enzyme inhibitor activity. The viscometric method of Bell *et al.* (1955), as modified by Porter *et al.* (1961), was still further modified to measure accurately enzyme preparations of high

activity and to correct for the decrease in enzyme activity that occurs during the analysis.

This decrease in enzyme activity was reduced by making up the enzyme to 0.0001 g/100 ml with 0.8% NaCl solution. The enzyme preparation used was Pectinol 10M (Rohm and Haas). Viscometric measurements were made at 5-min intervals in series of twelve. Standard enzyme-activity measurements (enzyme and substrate only) were run at the beginning and end of each series. An average of the two activity values was used, or values were interpolated for the appropriate 5-min intervals.

A further modification of the method was adoption of a half-hour period between the mixing of enzyme and inhibitor and their addition to the substrate, in order to allow the development of full inhibition. This was done because experimental tests indicated that the percentage inhibition increased from zero mixing time to 30 min prior to addition to substrate. The inhibitory power remained constant from 30 min to 3 hr.

To compare the activity of the isolated tannins with the activity of the original extract, each preparation from an 80-g sample was dissolved in water and made to 1000 ml. The water extracts, which had not been precipitated with caffeine, were also made to 1 L. Dilutions were prepared to give concentrations of each equivalent to 4 mg leaf/ml. The inhibitions obtained at this concentration were compared. The dilution was such that, at this concentration, any decrease in the activity of the preparation would produce large changes in the calculated inhibitory activity, as shown by Porter *et al.* (1961).

Paper chromatographic analysis. Many solvent variations were employed, using butanol, benzene, cresol, and toluene as the organic phase, water as the immobile phase, and acetic or *p*-toluene-sulfonic acid as the swamping acid. Ascending chromatography was on a small scale, using 6-inch squares of S & S no. 589 Blue Ribbon filter paper formed into cylinders. Dynel, polyvinylchloride, and glass-fiber supports, with butanol–acetic acid–water (4:1:5) as eluting solvent, were also employed. Ferric chloride, ultraviolet light, and ammoniacal silver nitrate were used for spot identification.

Hydrolytic methods. Samples of the isolated tannin were hydrolyzed by a method similar to that of Jurd (1956). The samples (0.5 g) were heated 22 hr at 100°C with 8 ml of 5% H_2SO_4. After cooling, the insoluble material was filtered and the filtrate extracted with ether.

RESULTS AND DISCUSSION

Description of isolated tannin. The solid obtained through the caffeine isolation and freeze-drying was pale tan and very light and bulky, became electrified on handling, and was surprisingly easy to keep dry. The yield (average of three 80-g portions) was 1.6% on a fresh basis. The fresh leaf contained 4.06% tannin as determined by the official hide-powder method of the American Leather Chemists' Association (1957). Recovery was 39.4%. Since the ALCA method measures all soluble materials removed by hide-powder under empirical conditions, it cannot be estimated what percentage would be precipitated by caffeine. The isolation is assumed to give relatively good recoveries of the tannin.

Activity of tannin isolates. All preparations, at concentrations equivalent to 4 mg leaf/ml, gave 97–98% inhibition, compared to 98–100% for the original extract. Since this concentration figure is on the slope of the activity-concentration curve, results of this order indicate that, in addition to fairly good recovery of the tannin, the isolation procedure also recovered 100% of the activity.

Stability of the inhibitor. The tannin inhibitor is quite stable. Measurements, by the viscometric method, on water extracts stored in a refrigerator for 1½ months still gave inhibitions of 98–100% at low dilutions. Solutions of the isolated tannins and of redissolved freeze-dried material, stored in a refrigerator for one month, also gave 100% inhibition.

Chemical and physical characteristics of the isolated tannins. Dialysis experiments using a cellophane membrane indicate a minimum molecular weight on the order of 10,000. Sedimentation studies show that there is a broad range of molecular weights. Light-scattering measurements gave an average molecular weight of 250,000. Therefore, the range is probably from about 10,000 to somewhere in the millions. Whether these high values are due to a range of discrete molecules or due to aggregation cannot be determined at this time. However, this may well explain the rather unique inhibitory power of these grape leaf tannins over other tannins of commercial value as hide tanning agents. According to White (1956), the

latter have molecular weights ranging between 600 and 2,000–3,000.

The original extracts behave essentially the same when chromatographed on paper using all of the solvents described and with the different support materials. One spot, containing essentially all of the material, remained at the starting position. Two other spots moved somewhat but were in very low concentration. A broad fluorescent streak was apparent in all chromatograms. The isolated tannins produced only one spot, which remained at the origin and produced a trace of streaking that fluoresced under ultraviolet light.

Samples of the isolated tannin produced red, insoluble phlobaphenes, almost equal to the weight of the original sample, when H_2SO_4 hydrolysis was attempted. The ether extract of the filtrate gave one strong spot and one weak spot when chromatographed on paper. The strong spot had the same R_f as gallic acid in all solvents, and produced the same color reactions with $FeCl_3$ and with ammoniacal $AgNO_3$. The ether extract also gave strong positive color tests for gallic acid with KCN. The aqueous layer from the ether extraction chromatographed to give one spot with the same R_f as glucose. Reaction on paper with ammoniacal $AgNO_3$ and *p*-anisidine hydrochloride gave identical results with those of pure glucose.

The tannin in Scuppernong grape leaves responsible for the inhibition of pectinase is a condensed type of extremely high average molecular weight. Acid treatment produces a high proportion of an extremely insoluble material (phlobaphenes). The presence of gallic acid and glucose in acid hydrolysates may be due to a slight hydrolysis of the condensed tannin, but may also be due to the presence of a trace of hydrolyzable tannin not easily separable from the major component.

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Mention of trade names in this paper does not imply endorsement by the U. S. Department of Agriculture.

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